REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED
April 02, 2003	Final Report	30 Sept 2001 - 29 Sept 2003

4. TITLE AND SUBTITLE

Assessment of PCB-dehalogenating Potential In Coastal Sediments

5a. CONTRACT NUMBER

5b. GRANT NUMBER
N00014-01-1-1053/1054

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kevin R. Sowers, PI
Harold D. May, co-PI
Joy E.M. Watts, co-PI

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Univ. of MD Biotechnology Institute, 701 E. Pratt St., Baltimore, MD 21202
Medical University of S. Carolina, 173 Ashley Ave, Charleston, SC 29425

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research, 800 N. Quincy St., Arlington, VA 22217-5000

10. SPONSOR/MONITOR'S ACRONYM(S)
ONR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AILABILITY STATEMENT
Distribution unlimited

20030508 127

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Molecular probes developed during the course of this ONR funded project were successfully used for both qualitative and quantitative assessment of PCB dechlorinating bacteria in PCB impacted sites. These probes are currently being used to determine the diversity of PCB dechlorianting bacteria required to dechlorinate commercial PCB mixtures and to assess the distribution of PCB dechlorinting species in PCB impacted sites of interest to the Navy.

15. SUBJECT TERMS
PCBs, bioremediation, marine sediments, natural attenuation, microbial catalysts

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Kevin R. Sowers
Unclass.	Unclass.	Unclass.	UP	4	19b. TELEPHONE NUMBER (Include area code) 410.234.8878

FINAL REPORT

GRANT #/PRINCIPAL INVESTIGATORS:
Kevin R. Sowers, Ph.D., Joy E.M. Watts, Ph.D., University of Maryland Biotechnology Institute, Center of Marine Biotechnology, 701 E. Pratt St., Baltimore, MD 21202
Award #: **N00014-01-1-1053**

Harold D. May, Ph.D.
Department of Microbiology & Immunology, Medical Univ. of S. Carolina
173 Ashley Avenue, POB #250504, Charleston, SC 29425
Award #: **N00014-01-1-1054**

GRANT TITLE: Collaborative Research: Assessment of PCB-biodehalogenating potential in coastal sediments

REPORT PERIOD: 30 September 2001 - 29 September 2002

OBJECTIVE: The overall objective of this research is to confirm the ability to use molecular probes as tools for assessing the PCB dehalogenating potential extant in coastal sediments. Studies outlined in this proposal will validate the application of molecular probes as a presumptive test for assessing PCB dechlorinating potential based on microbial enumeration coastal sediments. Ultimately the goal is to use molecular probes to monitor and assess the effectiveness of natural or bioaugmented PCB-dechlorination *in situ*.

APPROACH: The approach is as follows: (i) develop molecular probe assays for assessing PCB-dechlorinating potential in PCB impacted environments; (ii) screen for diversity and distribution of microbes that catalyze PCB dechlorination in selected environmental samples, (iii) assess the effects of sedimentary contaminants on PCB dechlorination by GC analysis and molecular probes assays. An effective monitoring assay combined with information on how dechlorination is affected by mixed contaminant wastes will provide fundamental information required to develop bioremedial strategies to promote intrinsic PCB transformation in impacted sites such as marine harbor sediments and dredge deposition sites.

Dr. Kevin Sowers (PI) and Dr. Joy Watts (co-PI), both of UMBI, developed the molecular screening tools used in this study. Dr. Harold May (PI), Dr. Qingzhong Wu, Mr. Greg Miller and Mr. Matt Brigmon, all of MUSC, have conducted the enrichment and isolation studies for identification of PCB dechlorinating bacteria and assessment of toxic effects of compounds on dechlorination.

ACCOMPLISHMENTS:
Objective 1. Development of molecular probe assays for assessing PCB-dechlorinating potential in PCB impacted environments. Assays for monitoring PCB dechlorinating bacteria in the environment had been precluded by the inability of laboratories to isolate and identify the microbial catalysts by conventional microbiological methods. With prior ONR supported research the PIs identified for the first time two sediment-free anaerobic PCB dechlorinating cultures by using an approach that combined classical enrichment protocols with screening of the microbial 16S rDNA communities (Pulliam Holoman et al., 1998; Watts et al, 2001, Cutter et al., 2001, and Wu et al., 2002). Using these cultures, primers were developed by obtaining 16S rDNA sequence for PCB dechlorinating microorganisms (o-17 and DF-1) in defined culture conditions, in our laboratory. These sequences were aligned using ClustalW (Higgins et al., 1996) with other closely related bacteria and regions of

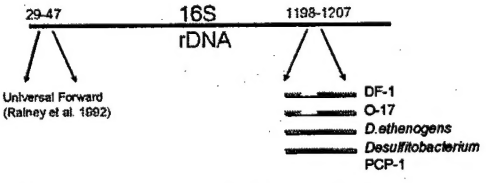

Figure 1. *Specific primer locations in 16S rDNA gene*

variability were examined for the suitability of probe formation. TCE and PCE dechlorinating microorganisms from the *Dehalococcoides ethenogens* genus were included (Fig. 1). It was important that the specific probes do not detect species of the *D.ethenogens* genus as this group has not been detected in PCB dechlorinating cultures. The specific primer was developed and tested *in silico* using the Genbank and RDP databases, the specific primer only produced high similarity values to *o*-17 and DF-1. The specific primer was paired with a universal forward primer (Rainey et al. 1992) and this combination yielded a product of 1198 bp. To examine the specificity of the primers, PCR reactions were performed with a wide range of other microbial DNA (Gram negative, Gram positive, Archaea and other known dechlorinators including *Desulfitobacteium* spp.). PCR reactions were optimized so that the primers generated PCR products with only the PCB dechlorinators *o*-17 or DF-1 in our assay (Fig. 2). To examine the PCR assay sensitivity microcosms were constructed containing 1 g of sediment and different amounts of o-17 16S rDNA. The total DNA was extracted from the sediment using the method described in Pulliam Holoman et al., 1996. Briefly sediment was treated by shaking in SDS lysis buffer with glass beads for 30s at a setting of 5.5 using a Fastprep FP120 instrument (ThermoSavant). DNA was extracted using a modified phenol chloroform procedure. The PCR assay was able to detect 16S rDNA easily for the microcosms containing 8500 ng/g - 85 ng/g, the band is faint but still visible in the 8.5 ng/g microcosm (Fig. 3). This experiment indicated that our detection level is at 8.5 ng/g 16S rDNA per microcosm. This level of sensitivity is within the commonly reported range for detecting microorganisms in environmental samples.

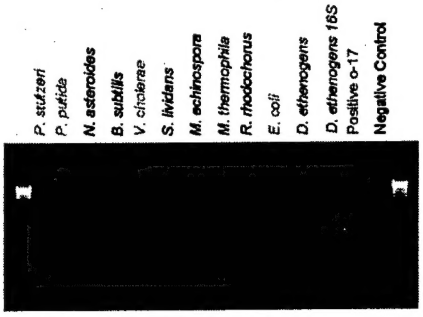

Figure 2. *PCR products with PCB-specific dechlorinator primers showing specificity for only PCB dechlorinator.*

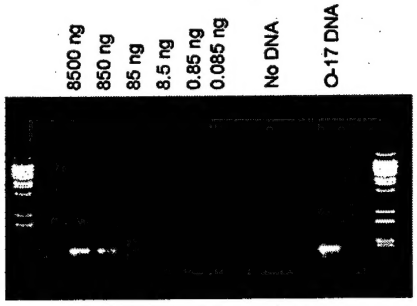

Figure 3. *PCR products with PCB-specific dechlorinator primers showing sensitivity with different amounts of DNA from the PCB dechlorinator o-17.*

Objective 2. Screening for diversity and distribution of microbes that catalyze PCB dechlorination in selected environmental samples. In order to optimize and assess the effectiveness of the probes for detecting PCB dechlorinating microorganisms in sediment, analyses were conducted with highly enriched sediment microcosms and with environmental samples impacted to varying degrees with PCBs.

PCB dechlorinating microcosms were started with 13 different PCB congeners and Aroclor 1260 (in triplicates with sterile controls), in addition to a set of control cultures without PCB. Baltimore Harbor sediments were added as inocula. The congeners are chosen on the basis that they are present in Aroclor 1260 by more than 3 weight % according to Frame et al. (1996) and that Baltimore Harbor sediments have the capacity to dechlorinate these congeners (Wu et al., 1997). After 8 months of incubation cultures with 6 of the congeners showed dechlorination. The pattern of this dechlorination is seen in Fig. 4. Most of the dechlorination is occurring in the *meta* position, but there is evidence of both *para* and *ortho* dechlorination. These cultures have been transferred and will be assayed again to confirm the pathways of dechlorination. Preliminary analysis with the specific probe of the cultures enriched with PCB 151 detected three 16S rDNA genes with highest sequence similarity to PCB dechlorinator *o*-17. Since most of the funding period was required to develop the cultures, studies utilizing these cultures will be

continued during the course of a subsequently funded ONR project (N00014-03-1-0035). However, the preliminary results suggest that this is a valid approach for determining which microorganisms catalyze specific patterns of PCB dechlorination. Ultimately this experiment will enable us to determine the diversity of PCB dechlorinating species required to dechlorinate a commercial PCB mixture (e.g., Aroclor 1242, 1260, etc.).

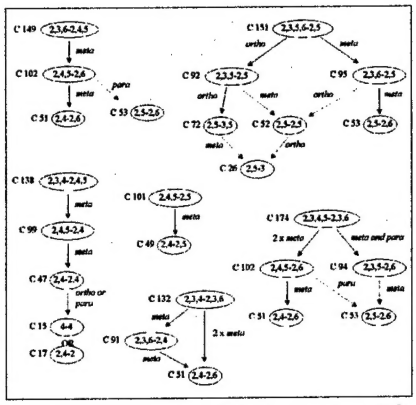

Figure 4. *PCB dechlorinating pathways established in microcosms with Baltimore Harbor sediment.*

Analysis of PCB dechlorinating cultures revealed that phylogenetically this group appeared to be distinct from other dechlorinating bacteria (Fig. 5). The Dehalococcoides group has the highest sequence similarity to the PCB dechlorinators and from our analysis they form a distinct and separate group (cultured isolates and non-isolated 16S sequences). To examine environmental samples Baltimore Harbor, sediment was collected from a number of sites within the bay. DNA was extracted and subjected to the specific PCR. PCR products were cloned, sequenced and analyzed from sites in the Inner Harbor; no PCR products were detected from the Outer Bay samples. These sequences appeared to be closely related to the laboratory PCB dechlorinating enrichment cultures although distinct from the *D. ethenogens* dechlorinating group (see Figure 5). Although this assay is presumptive, as 16S rDNA does not infer functional activity, it is interesting that Baltimore Inner Harbor (a highly contaminated PCB site) contained closely related sequences to PCB dechlorinating microorganisms in the laboratory. However, from the less impacted Outer Harbor region no specific PCR products were detected indicating that these sequences may be linked to the presence / absence of PCBs in the environment.

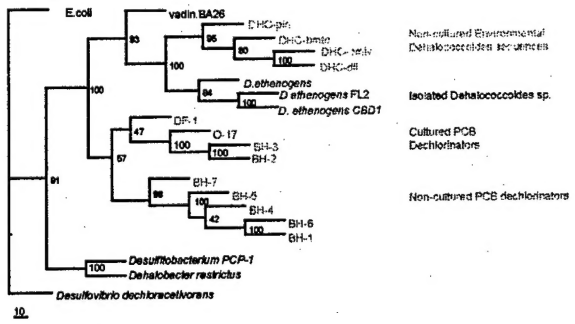

Figure 5. *Phylogenetic tree generated using the neighbor-joining (Saitou and Nei, 1987) algorithm in the PHYLIP software (Felsenstein, 1993). Distance matrices for the neighbour-joining and Fitch and Margoliash methods generated as described by Jukes and Cantor (1969). Numbers represent 1000 bootstrap resamplings of neighbour-joining data.*

Objective 3. Assessing the effects of sedimentary contaminants on PCB dechlorination by GC analysis and molecular probes assays. Effects of various known environmental organic and inorganic co-contaminants of PCBs were tested on PCB dechlorinator DF-1. Among the organic compounds tested two classes of organochlorines (chlorobenzenes CBZ and chlorinated ethenes CE) support growth and dechlorinating activity. Further analysis of the dechlorinating capabilities of DF-1, show that this microorganisms is capable of dechlorinating hexa-CBZ → penta-CBZ → 1,2,3,5-CBZ → 1,3,5-CBZ (Fig. 6), which is the predominant pathway reported in microcosm studies. *This was the first identification of an organism that could complete this CBZ pathway and was the first observation linking PCB and CBZ dechlorination to the same organism.* Several other organic co-contaminants, including several organochlorines, which occur in the environment from biogenic, sources such as fungi were not attacked by the PCB dechlorinating culture. More recently the PIs have discovered that DF-1 dechlorinates PCE to trichloroethene (TCE) and *cis* and *trans* dichloroethene

(cDCE and tDCE) (unpublished, see below). For the first time dechlorination of these three different classes of organochlorines has been demonstrated with one organism. The effects of the chromium, copper, lead and zinc, contaminants currently found in Baltimore Harbor, were tested to determine the effects of these heavy metals on the dechlorinating activity of DF-1. Cultures were sampled over the course of 6 months and analyzed for activity. Preliminary results at the time of this report are inconclusive because the cultures are not fully developed, but the cultures will continue to be monitored as they mature and the results reported in the current ONR-funded project.

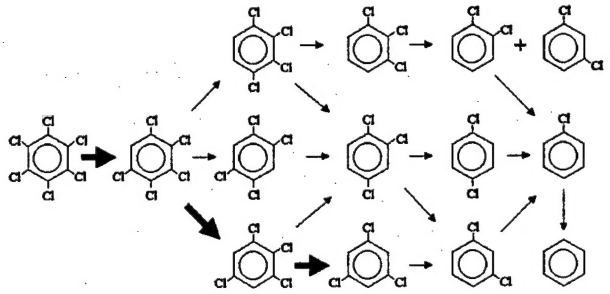

Figure 6. Microbial pathways of chlorobenzene reductive dechlorination. The most frequently cited predominant pathway, and the path performed by DF-1, is marked with large arrows.

CONCLUSIONS: For the first time molecular probes have been developed for assessment of presumptive dechlorinating potential in PCB impacted sites. Preliminary results indicate that PCB dechlorinating populations are only detected in PCB impacted sites. Results also suggest that putative PCB dechlorinating microbes have highest similarity with the known PCB declorinating species DF-1 and *o*-17 and that they cluster in a discreet phylogenetic branch. PCB dechlorinating microorganisms also have the ability to utilize the chloroethenes and chlorobenzenes, which often occur as co-contaminants of PCBs.

SIGNIFICANCE: Molecular probes developed during the course of this ONR funded project were successfully used for both qualitative and quantitative assessment of PCB dechlorinating bacteria in PCB impacted sites. These probes are currently being used to determine the diversity of PCB dechlorinating bacteria required for the breakdown of commercial PCB mixtures and to assess the distribution of PCB dechlorinating species in PCB impacted sites of interest to the Navy.

PATENT INFORMATION: Information from this project contributed to a patent that relates to the use of microbes to catalyze dechlorination of PCBs.

AWARD INFORMATION: None.

PUBLICATIONS:

1. Nicholas J. Drenzek, Christopher M. Reddy, Timothy I. Eglinton, Carl O. Wirsen, Harold D. May, Neil C. Sturchio, Linnea J. Heraty, Kevin R. Sowers, and Qingzhong Wu. Invariant Chlorine Isotopic Signatures During PCB Reductive Dechlorination. *Submitted.*

2. Wu, Q., G.P. Meier, K.R. Sowers, and H.D. May. 2002. Reductive dechlorination of polychlorinated benzenes by Bacterium DF-1, a polychlorinated biphenyl-dechlorinating microorganism. *Environ. Sci. & Technol. 36:* 3290-3294.

3. Wu, Q., Watts, J.E.M., K.R. Sowers and H.D. May. 2002. Identification of a bacterium that catalyzes double-flanked PCB dechlorination. *Appl. Environ. Microbiol. 69(2):* 807-812.